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USE OF AN ENTEROBACTERIUM OmpA PROTEIN FOR SPECIFIC TARGETING TO ANTIGEN-PRESENTING CELLS

The invention relates to the use of an enterobacterium OmpA protein, preferably the Klebsiella *pneumoniae* P40 protein, for specific targeting of a biologically active substance which is associated with it to antigen-presenting cells, in particular human dendritic cells. The invention also relates to the use of the OmpA protein for preparing a pharmaceutical composition intended for the prevention and/or treatment of diseases, in particular cancers associated with a tumor antigen, autoimmune diseases or infectious diseases.

Vaccination is an effective means of preventing or attenuating viral or bacterial infections. The success of vaccination campaigns in this domain has made it possible to extend the vaccine concept to other domains, such as that of cancer and of autoimmune diseases. With regard, for example, to certain forms of cancer, the ineffectiveness of conventional therapies and/or their side effects, such as chemotherapy or radiotherapy, has prompted the search for alternative therapy. Thus, specific tumor antigens expressed at the surface of tumor cells can be used as a target in immunotherapy for the elimination of these cells. One of the major problems commonly encountered in preparing these vaccines is that the vaccine antigens, when they are administered alone to the host, are not immunogenic enough to induce an immune response which is sufficiently effective to confer the desired protection. These antigens are thus often covalently coupled to a carrier molecule such as, for example, an epitope of the diphtheria toxin, the tetanus anatoxin (TT), a surface antigen of the hepatitis B virus, the VP1 antigen of the poliomyelitis virus or any other toxin, or viral or bacterial antigen, such as antigenic proteins derived from the enterobacterium external

membrane, which have the property of potentiating the immune response (humoral or cellular) of the antigen which is associated with it, for instance the OmpA protein named P40 derived from Klebsiella *pneumoniae* (described in international patent applications WO 95/27787 and WO 96/14415). However, in most cases, another component has proved to be necessary in order to increase the effectiveness of the vaccine and, currently, the only adjuvant authorized in humans is alum.

Through immunology, it has recently been discovered that dentritic cells (DCs) play a major role in the immune system. These cells, derived from bone marrow stem cells, are professional antigen-presenting cells involved in the antigen-specific primary immune response (Peters J. et al., 1996). They ingest or internalize antigens and present the fragments of these antigens to naïve T cells. This ingestion induces, at the surface of the dendritic cells, the expression of costimulation molecules such as CD80 and CD86. These molecules allow close interaction with T cells (Girolomoni G. and Ricciardi-Castagnoli P., 1997, Immunol. Today, 18, 102-104). Dendritic cells are distributed diffusely in tissues. They are found in the skin and lymphoid organs (Hinrich J. et al., 1996, Immunol. Today, 17, 273-277).

Due to their effectiveness in presenting antigens and in stimulating the immune system, dendritic cells have been used to generate antiviral (Ludewig B. et al., 1998, J. Virol., 72, 3812-3818; Brossard P. et al., 1997, J. Immunol., 158, 3270-3276) or anticancer (Nestle F.O. et al., 1998, Nat. Med., 4, 328-332) cytotoxic CTL responses. Approaches have consisted in loading dendritic cells *ex vivo* with the antigen of interest (peptides or cell lysate) and reimplanting these cells in the patient. Other approaches consist in transfecting dendritic cells *ex vivo* with the gene encoding the antigen of interest and in reinjecting these transfected cells (Gilboa E. et

al., 1998, Cancer Immunol. Immunother., 46, 82-87). These approaches have been used successfully in mice and recently in humans (Hsu F.J. et al., 1996, Nat. Med., 2, 52-58). Dendritic cells loaded with antigens present the peptides via class I or II molecules, and induce the activation of CD4 or CD8+ T lymphocytes. Consequently, the possibility of directing the antigens chosen, such as proteins or polysaccharides, or viral vectors capable of transferring genes encoding these antigens, toward dendritic cells would make it possible to improve the effectiveness of immune system stimulation. In addition, specific targeting of antigen-presenting cells (APCs), in particular dendritic cells, would make it possible to avoid the steps of removal, of purification and of *ex vivo* treatment of autologous or heterologous APCs with the tumor antigens or the viral vectors, and the reimplantation of the treated APCs.

In order to specifically target dendritic cells with active substances of interest, such as proteins or viral vectors capable of transferring genes encoding these proteins of interest, many studies have consisted in identifying molecules which would bind preferentially to the dendritic cells, or receptors which would be expressed specifically on the dendritic cells. A receptor DEC 205, involved in the treatment of the antigen, has been identified on murine (Jiang W. et al., 1995, Nature, 375, 151-155) and human (Kato M. et al., 1998, Immunogenetics, 47, 442-450) dendritic cells. The analysis of the structure of this receptor reveals carbohydrate-recognition domains which are thought to be involved in the capture, internalization and/or presentation of antigens carrying carbohydrate residues. However, the authors give no information concerning the ligands which can be bound by this receptor. On the other hand, the authors mention that the carbohydrate-recognition domains of the receptor DEC-205 which are thought to be involved in the capture, internalization and/or presentation of

antigens (cysteine-rich domains) are also present in more than 50 proteins, including some cell receptors.

Thus, there exists, today, a need for a compound which is capable of specifically targeting an antigen-presenting cell (APC), in particular a dendritic cell, and which is also capable of being internalized by said cell. Such a compound capable of binding specifically to these cells, and then of being internalized, would have the advantage of being able to be used as a compound for the transport and targeting of a biologically active substance, the effectiveness of which is modified by and/or linked to the binding and/or the internalization of this substance by these cells. In addition, it would be advantageous if this compound being sought could be easily associated with the active substance by chemical coupling or by coupling resulting from genetic fusion, or if it could be expressed at the surface of a host cell or at the surface of a viral particle for the transfer of a gene of interest into these APCs.

The authors of the present invention have demonstrated, surprisingly, that an enterobacterium external membrane protein of OmpA type, in particular the Klebsiella *pneumoniae* P40 protein, is capable not only of binding specifically to an APC, but also capable of being internalized by said APC, in particular by a dendritic cell.

Thus, the present invention relates to the use of an enterobacterium OmpA protein, or of a fragment thereof, for specific targeting of a biologically active substance which is associated with it to antigen-presenting cells.

In the present invention, the expression "antigen-presenting cells" will be intended to refer to professional APCs which form an integral part of the immune system, such as dendritic cells, macrophages, B lymphocytes or monocytes.

In the present invention, the term "protein" will also be intended to refer to peptides or

polypeptides, and the term "OmpA" (for "Outer Membrane Protein") will be intended to refer to external membrane proteins of type A.

The expression "fragment of an OmpA protein" is intended to refer to any fragment of amino acid sequence included in the amino acid sequence of the OmpA protein capable of binding specifically to APCs, in particular dendritic cells, and comprising at least 5 amino acids, preferably 10 amino acids, or more preferably 15 amino acids, said fragments also being capable of being internalized into said APCs.

The expression "biologically active substance" is intended to refer to any compound which is capable of exercising therapeutic activity and the activity of which can be modified via APCs. Mention may be made, as an example of such biologically active substances, but without being limited thereto, of immunogenic compounds such as antigens or haptens which are protein, poly- or oligosaccharide, glycoprotein or lipoprotein in nature, or in general of organic origin, these immunogenic compounds possibly being carried by complex structures such as bacteria or viral particles.

The expression "biologically active substance" is also intended to refer to any compound capable of modifying the functional activity of APCs, in particular the growth, differentiation or system of expression thereof. Mention may be made, as an example of such biologically active substances, but without being limited thereto, of cellular growth factors including cytokines (IL-4, IL-3, GM-CSF, TNF-α), and nucleic acids which encode homologous or heterologous proteins of interest and which are capable of being expressed by APCs.

A subject of the invention is also the use of an enterobacterium OmpA protein, or of a fragment thereof, according to the invention, characterized in that said enterobacterium OmpA protein, or a fragment thereof, binds specifically to antigen-presenting cells, and in that said enterobacterium OmpA protein,

or a fragment thereof, is internalized into the antigen-presenting cells.

Preferably, the invention comprises the use of an enterobacterium OmpA protein, or of a fragment thereof, according to the invention, characterized in that said antigen-presenting cells are chosen from dendritic cells, monocytes and B lymphocytes, more preferably dendritic cells.

In a particular embodiment, the invention comprises the use of an enterobacterium OmpA protein, or of a fragment thereof, according to the invention, characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained from a culture of said enterobacterium, using an extraction process.

Processes for extraction of bacterial membrane proteins are known to those skilled in the art and will not be developed in the present description. Mention may be made, for example, but without being limited thereto, of the extraction process described by Hauew J.H. et al. (Eur. J. Biochem, 255, 446-454, 1998).

In another preferred embodiment, the invention also comprises the use of an enterobacterium OmpA protein, or of a fragment thereof, according to the invention, characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained by recombinant process.

Methods for preparing recombinant proteins are today well known to those skilled in the art and will not be developed in the present description; reference may, however, be made to the method described in the examples. Among the cells which can be used for producing these recombinant proteins, it is of course necessary to mention bacterial cells (Olins P.O. and Lee S.C., 1993, Recent advances in heterologous gene expression in E. coli. Curr. Op. Biotechnology 4:520-525), but also yeast cells (Buckholz R.G., 1993, Yeast Systems for the Expression of Heterologous Gene

Products. Curr. Op. Biotechnology 4:538-542), as well as animal cells, in particular cultures of mammalian cells (Edwards C.P. and Aruffo A., 1993, Current applications of COS cell based transient expression systems. Curr. Op. Biotechnology 4:558-563), and also insect cells in which it is possible to use processes implementing baculoviruses for example (Luckow V.A., 1993, Baculovirus systems for the expression of human gene products. Curr. Op. Biotechnology 4:564-572).

Most preferably, the use according to the invention is characterized in that said enterobacterium is Klebsiella *pneumoniae*.

In particular, the invention relates to the use according to the invention, characterized in that the amino acid sequence of said Klebsiella *pneumoniae* OmpA protein, or a fragment thereof, comprises:

a) the amino acid sequence having the sequence SEQ ID No 2;

b) the amino acid sequence of a sequence having at least 80%, preferably at least 85%, 90% or 95%, homology with the sequence SEQ ID No 2; or

c) the amino acid sequence of a fragment, of at least 5 amino acids, of a sequence as defined in a) or b).

The expression "sequence having at least 80%, preferably at least 85%, 90% or 95%, homology with the reference sequence SEQ ID No 2" is intended to refer to an amino acid sequence having a degree of identity, after optimal alignment, of at least 80%, 85%, 90% or 95%, respectively, with the reference sequence SEQ ID No 2, said homologous sequence, or a said fragment thereof of at least 5 amino acids as defined above in c), being characterized in that it binds specifically to antigen-presenting cells and, where appropriate, in that it is internalized into the antigen-presenting cells.

For the purpose of the invention, the expression "percentage of identity" between two nucleic acid or amino acid sequences is intended to refer to a percentage of nucleotides or of amino acid residues

which are identical between the two sequences to be compared, obtained after the best alignment, this percentage being purely statistical and the differences between the two sequences being distributed randomly and throughout their length. The best alignment or optimal alignment is the alignment for which the percentage of identity between the two sequences to be compared, as calculated hereinafter, is highest. Sequence comparisons between two nucleic acid or amino acid sequences are conventionally carried out by comparing these sequences after having aligned them optimally, said comparison being carried out by segment or by "window of comparison", so as to identify and compare local regions of sequence similarity. The optimal alignment of the sequences for comparison can be produced, other than manually, by means of the local homology algorithm of Smith and Waterman (1981) [Ad. App. Math. 2:482], by means of the local homology algorithm of Neddleman and Wunsch (1970) [J. Mol. Biol. 48:443], by means of the similarity search method of Pearson and Lipman (1988) [Proc. Natl. Acad. Sci. USA 85:2444], by means of computer software using these algorithms (GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI, or BLASTN or BLASTX, Altschul et al., J. Mol. Biol. 215, 403, 1990).

The percentage of identity between two nucleic acid or amino acid sequences is determined by comparing these two optimally aligned sequences by window of comparison in which the region of the nucleic acid or amino acid sequence to be compared can comprise additions or deletions with respect to the reference sequence for optimal alignment between these two sequences. The percentage of identity is calculated by determining the number of identical positions for which the nucleotide or amino acid residue is identical between the two sequences, dividing this number of identical positions by the total number of positions in the window of comparison, and multiplying the result

obtained by 100 so as to obtain the percentage of identity between these two sequences.

The invention also comprises the use according to the invention, characterized in that said biologically active substance is chosen from proteins or peptides, lipopeptides, polysaccharides, oligosaccharides, nucleic acids, lipids and chemical substances.

A subject of the present invention is also the use of an enterobacterium OmpA protein, or of a fragment thereof, according to the invention, characterized in that said biologically active substance is coupled by covalent attachment with said OmpA protein, or a fragment thereof, in particular by chemical coupling.

In a particular embodiment, the use according to the invention is characterized in that one or more attachment elements is (are) introduced into said OmpA protein, or a fragment thereof, and/or into said biologically active substance, in order to facilitate the chemical coupling; preferably said attachment element introduced is an amino acid.

According to the invention, it is possible to introduce one or more attachment elements, in particular amino acids, in order to facilitate the coupling reactions between the OmpA protein, or a fragment thereof, and the biologically active substance, such as an antigen or a hapten. The covalent coupling between the OmpA protein, or a fragment thereof, and the biologically active substance, such as an antigen or a hapten, according to the invention can be carried out at the N- or C-terminal end of the OmpA protein, or a fragment thereof. The bifunctional reagents which allow this coupling will be determined as a function of the end of the OmpA protein, or a fragment thereof, chosen to perform the coupling, and on the nature of the biologically active substance to be coupled.

In another particular embodiment, the use according to the invention is characterized in that said biologically active substance coupled by covalent attachment with said OmpA protein, or a fragment thereof, is a recombinant chimeric protein resulting from the expression of a nucleic acid construct encoding said biologically active substance and said OmpA protein, or a fragment thereof.

The conjugates derived from coupling to said OmpA protein, or a fragment thereof, can be prepared by genetic recombination. The chimeric or hybrid protein (conjugate) can be produced using recombinant DNA techniques, by insertion into or addition to the DNA sequence encoding said OmpA protein, or a fragment thereof, of a sequence encoding said biologically active substance which is protein in nature.

The processes for synthesizing the hybrid molecules encompass the methods used in genetic engineering for constructing hybrid polynucleotides encoding the desired polypeptide sequences. Reference may, for example, be advantageously made to the technique for obtaining genes encoding fusion proteins, described by D.V. Goeddel (Gene expression technology, Methods in Enzymology, vol. 185, 3-187, 1990).

The invention relates most particularly to the use of an enterobacterium OmpA protein, or of a fragment thereof, according to the invention, characterized in that said biologically active substance is an antigen or a hapten.

In another aspect, the invention relates to the use of an enterobacterium OmpA protein, or of a fragment thereof, according to the invention, for modifying the immune response against an antigen or a hapten, preferably for improving the immune response against an antigen or a hapten.

The invention also comprises the use of an enterobacterium OmpA protein, or of a fragment thereof, according to the invention, for preparing a pharmaceutical composition intended to prevent or to

treat a disease with an active substance, the effectiveness of which is modified by and/or linked to the internalization thereof by antigen-presenting cells, preferably by dendritic cells.

Preferably, the use according to the invention is related to the preparation of a pharmaceutical composition intended to prevent or to treat cancers, preferably cancers associated with a tumor antigen, autoimmune diseases, allergies, graft rejections, cardiovascular diseases, diseases of the central nervous system, inflammatory diseases, infectious diseases or diseases linked to an immunodeficiency.

A subject of the invention is in particular the use of an enterobacterium OmpA protein, or of a fragment thereof, according to the invention, for preparing a pharmaceutical vaccine composition intended to prevent or to treat an infectious disease or a cancer associated with a tumor antigen.

The invention also comprises the use according to the invention, characterized in that said pharmaceutical composition also comprises an adjuvant which promotes the immune response, such as alum.

The invention also comprises the use according to the invention, characterized in that said pharmaceutical composition is vehicled in a form which makes it possible to improve the stability and/or the immunogenicity thereof, in particular in the form of a liposome, of a viral vector or of a transformed host cell capable of expressing a recombinant chimeric protein resulting from the expression of a nucleic acid construct encoding said biologically active substance and said OmpA protein, or a fragment thereof.

The legends of the figures and examples which follow are intended to illustrate the invention without in any way limiting the scope thereof.

Legends of the figures:

Figure 1: Binding of rP40-Alexa to various cell types. After incubation of rP40-Alexa on various cell types, the specific binding of rP40-Alexa (bold line) is

measured by flow cytometry. The binding of a nonrelevant protein (glycophorin) is represented with a fine line.

Figure 2: Influence of the concentration of rP40 on the binding to dendritic cells.

Figure 3: Inhibition of the binding of rP40-Alexa to dendritic cells, with unlabeled rP40.

After incubation of dendritic cells with various concentrations of unlabeled rP40, rP40-Alexa is added. The binding of rP40-Alexa is quantified by flow cytometry.

Figure 4: Evaluation of the binding of various labeled proteins to dendritic cells.

P40, TT (tetanus anatoxin) and BB (derived from the streptococcus G protein) carrier proteins labeled with Alexa are incubated with dendritic cells (thick line). A nonrelevant protein is used as a negative control (fine line). The binding is measured by flow cytometry.

Figure 5A and 5B: Internalization of rP40-Alexa into dendritic cells.

After incubation of dendritic cells with rP40-Alexa at 4°C (left-hand panel, figure 5A) or at 37°C (right-hand panel, figure 5B), the cells are observed by confocal microscopy (x 220 magnification).

Example 1: Cloning of the rP40 gene

The gene encoding the recombinant P40 protein, named rP40, was obtained by PCR amplification using the genomic DNA of Klebsiella *pneumoniae* IP I145 (Nguyen et al., Gene, 1998). The coding gene fragment of rP40 is inserted into various expression vectors, in particular a vector under the control of the Trp operon promoter. The amino acid sequence of the rP40 protein and the nucleotide sequence encoding the P40 protein are represented by the sequences SEQ ID No 2 and SEQ ID No 1, respectively, in the sequence listing hereinafter.

An *E. coli* K12 producer strain was transformed with an expression vector pvaLP40. The rP40 protein is

produced in the form of inclusion bodies with a significant yield (> 10%, g of proteins/g of dry biomass). This example is only an illustration of the expression of rP40, but it may be extended to other bacterial strains, and also to other expression vectors.

Example 2: Process for fermenting rP40 fusion proteins

An Erlenmeyer containing 250 ml of TSB (Tryptic Soy Broth, Difco) medium containing ampicillin (100 μg/ml, Sigma) and tetracycline (8 μg/ml, Sigma) is inoculated with the recombinant *E. coli* strain described above. The incubation is carried out overnight at 37°C, and then 200 ml of this culture is used to seed 2 liters of culture medium in a fermenter (Biolafitte, France). In a quite conventional way, the culture medium can be composed of chemical agents, supplemented with vitamins and/or yeast extracts, known to have a growth at high density of bacterial cells.

The parameters controlled during the fermentation are: the pH, the stirring, the temperature, the level of oxygenation and the supply of combined sources (glycerol or glucose). In general, the pH is regulated at 7.0 and the temperature is fixed at 37°C. The growth is controlled by supplying glycerol (87%) at a constant flow rate (12 ml/h) so as to maintain the dissolved oxygen tension signal at 30%. When the turbidity of the culture (measured at 580 nm) reaches the value of 80 (after approximately 24 hours of culturing), the protein production is triggered by adding indole acrylic acid (IAA) at the final concentration of 25 mg/l. Approximately 4 hours after induction, the cells are harvested by centrifugation. The amount of wet biomass obtained is approximately 200 g.

Example 3: Process for extracting and for purifying the rP40 protein

Extraction of rP40

After centrifugation of the culture broth (4000 rpm, 10 min, 4°C), the cells are resuspended in a 25 mM Tris-HCl buffer, pH 8.5. The insoluble substances or inclusion bodies are obtained after treatment with lysozyme (0.5 g/liter, 1 hour at room temperature / gentle stirring). The inclusion body pellet obtained by centrifugation (50 min at 10 000 g at 4°C) is taken up in a 25 mM Tris-HCl buffer at pH 8.5, containing 5 mM $MgCl_2$, and then centrifuged (15 min at 10 000 g).

The inclusion bodies are solubilized at 37°C for 2 hours in a 25 mM Tris-HCl buffer, pH 8.5, containing 7 M urea (denaturing agent) and 10 mM dithiothreitol (reduction of disulfide bridges). Centrifugation (15 min at 10 000 g) makes it possible to eliminate the insoluble particles.

Thirteen volumes of 25 mM Tris-HCl buffer, pH 8.5, containing NaCl (8.76 g/l) and Zwittergent 3-14 (0.1%, w/v) are then used to resuspend. The solution is left overnight at room temperature with gentle stirring, in contact with the air (promotes the renaturation of the protein by dilution and reoxidation of the disulfide bridges).

Purification of the rP40 protein

Anion exchange chromatography step.

After a further centrifugation, the solution is dialyzed against a 25 mM Tris-HCl buffer, pH 8.5, containing 0.1% Zwittergent 3-14 (100 X volumes of buffer) overnight at 4°C.

The dialysate is loaded onto a column containing a support of strong anion exchange type (Biorad Macro Prep High Q gel), equilibrated in the buffer described above, at a linear flow rate of 15 cm/h. The proteins are detected at 280 nm. The rP40 protein is eluted, with a linear flow rate of 60 cm/h, for a concentration of NaCl of 0.2 M in the 25 mM Tris-HCl, pH 8.5, 0.1% Zwittergent 3-14 buffer.

Cation exchange chromatography step

The fractions containing the rP40 protein are pooled and concentrated by ultrafiltration with the aid of an Amicon stirring cell system used with a Diaflo membrane of YM10 type (cut-off threshold 10 kDa), for volumes of about 100 ml, or with the aid of a millipore Minitan tangential-flow filtration system used with membrane plates having a cut-off threshold of 10 kDa, for larger volumes. The fraction thus concentrated is dialyzed overnight at 4°C against a 20 mM citrate buffer, pH 3.0, containing 0.1% of Zwittergent 3-14.

The dialysate is loaded onto a column containing a support of strong cation exchange type (Biorad Macro Prep High S gel), equilibrated in the 20 mM citrate buffer, pH 3.0, containing 0.1% of Zwittergent 3-14. The rP40 protein is eluted (rate 61 cm/h) for a concentration of NaCl of 0.7 M. The electrophoretic profiles show a degree of purity of about 95%. The condition of the protein is monitored by SDS-PAGE. The P40 protein extracted from the Klebsiella *pneumoniae* membrane has a characteristic electrophoretic behavior (migration) according to its denatured or native form. The native form (β-sheet structure) in fact has a lower molecular mass than the form denatured (α-helix structure) under the action of a denaturing agent, such as urea or guanidine hydrochloride, or with heating at 100°C in the presence of SDS. The rP40 protein is not correctly renatured at the end of renaturation, whether this renaturation is carried out in the presence or absence of 0.1% (w/v) Zwittergent 3-14. On the other hand, total renaturation is obtained after dialysis against a 25 mM Tris/HCl buffer, pH 8.5, containing 0.1% (w/v) of Zwittergent 3-14. However, it should be noted that this renaturation is obtained only when the dilution step and the treatment at room temperature are, themselves, carried out in the presence of Zwittergent 3-14 (negative results in the absence of detergent).

Example 4: Specific binding of rP40 to antigen-presenting cells (APCs). Methodology

Purification of human T lymphocytes

Mononucleated cells (MNCs) are isolated from the peripheral blood of healthy volunteers, by centrifugation (1800 rpm, 20 min, room temperature), on a Ficoll gradient. After centrifugation, the MNCs, located at the ficoll/plasma interface, are harvested and washed twice with complete culture medium (CM) (RPMI 1640 + 10% FCS + L-glutamine + antibiotic). The T lymphocytes are then isolated by the rosetting technique, which uses their capacity to bind to sheep red blood cells (SRBCs). Briefly, the MNCs are incubated with SRBCs for 1 hour at 4°C. After centrifugation on a ficoll gradient, the B lymphocytes and monocytes are located at the interface, whereas the T lymphocytes bound to the SRBCs are in the cell pellet. After recovery of the cell pellet and lysis of the SRBCs with a hypotonic saline solution, the purity of the T lymphocytes is assessed by flow cytometry with an anti-CD3 antibody, and is greater than 95%.

Purification of the human monocytes

The monocytes are purified from the MNCs by positive selection using MACS (Magnetic Activated Cell Sorter) technology. The MNCs are labeled with an anti-CD14 antibody coupled to magnetic particles, and then passed over a magnetized column. The monocytes to which the antibody-colloid complexes are bound remain in the column, whereas the cells which have not bound the antibody are eluted with successive washes. Next, the monocytes are detached by performing washes in the absence of magnet. The purity of the fraction collected is greater than 98%.

Generation of human dendritic cells (DCs) from monocytes

The purified monocytes are cultured at the concentration of 106/ml in CM for 6 to 7 days, in the presence of IL 4 (20 ng/ml) and of CMCSF (20 ng/ml). The DCs generated at this stage are immature DCs which

express CDIa and no, or relatively little, CD83. Their phenotype is verified using the flow cytometry technique.

Purification of human B lymphocytes from tonsils

The tonsils are ground, and the cells harvested are loaded onto a ficoll gradient. The MNCs recovered at the interface are washed and then incubated with SRBCs. After ficoll, the B lymphocytes are located at the interface, whereas the T lymphocytes bound to the SRBCs are in the cell pellet. The B lymphocytes are then washed. Their purity, verified by flow cytometry, is greater than 96%.

Culturing of cell lines

The RPMI 8866, DAUDI, HL60 and Jurkat cell lines are cultured in CM.

Coupling of rP40 to the Alexa488 fluorochrome

The concentration of the rP40 protein is adjusted to 2 mg/ml in PBS. 50 µl of 1 M sodium bicarbonate are added to 500 µl of the protein. The solution is then transferred into a reaction tube containing the Alexa488 dye and the coupling takes place at room temperature. After 1 h, the coupling reaction is stopped by adding 15 µl of hydroxylamine. The labeled protein is separated from the free dye by column purification.

The amount of rP40 labeled with Alexa488 is then estimated by colorimetric assay.

- Study of the binding of p40-Alexa488 to the various cells, by flow cytometry.

For each labeling, 200 000 cells are washed with FACS buffer (PBS + 1% BSA + 0.01% sodium azide) and resuspended, in a cone-bottomed 96-well plate, in 50 µl of FACS buffer. The P40-Alexa488 protein or the control protein (glycophorin-Alexa488) are then added at 10^{-6}M for approximately 1 h at 4°C. After incubation, the cells are then washed 3 times with FACS buffer, and then resuspended in 200 µl of this same buffer and analyzed by flow cytometry.

Result

The rP40 protein binds selectively to human APCs such as:

- the monocytes derived from human blood,
- the dendritic cells generated from the peripheral blood monocytes,
- the B lymphocytes derived from tonsils, the B-lymphocyte lines: DAUDI and RPMI 8866 (cf. fig. 1) and the B lymphocytes derived from peripheral blood (result not shown).

No binding is observed to cells which do not have the capacity to present antigens, such as nonactivated peripheral blood T lymphocytes, the nonactivated Jurkat T-lymphocyte line and the nonactivated HL60 monocyte line.

Example 5: The binding of rP40 to the DCs is specific

1) The binding of rP40 to the DCs is dose-dependent.

Method

200 000 DCs are washed with FACS buffer and incubated in 50 µl of buffer in the presence of various concentrations of rP40 (from 10^{-10} to 5×10^{-6} M) for approximately 1 hour at 4°C. After incubation, the cells are washed 3 times with FACS buffer, and then resuspended in 50 µl of this same buffer containing 5 µg/ml of an anti-P40 rabbit polyclonal antibody or of a control rabbit IgG antibody. After incubation for 20 minutes, the cells are rewashed and incubated in 100 µl of FACS buffer containing a floresceine-labeled anti-rabbit IgG goat polyclonal antibody (diluted to 1:200). After incubation for 20 minutes, the cells are washed, taken up in FACS buffer and analyzed by flow cytometry.

Result

The binding of rP40 to the DC is significant from 10^{-7} M ($p<0.001$) and at a maximum at 2×10^{-6} M (cf. fig. 2).

2) Unlabeled rP40 protein decreases the binding of rP40 Alexa488 to the DCs.

Method

In order to demonstrate the specificity of the binding of P40, competition is carried out between rP40-Alexa488 and unlabeled rP40. The DCs were incubated for 10 minutes with 5×10^{-8} to 2×10^{-6} M of unlabeled rP40, and then P40-Alexa488 (used at 2×10^{-6} M) was added. After incubation for 20 minutes at 4°C, the cells were analyzed by flow cytometry as described previously.

Result

The unlabeled rP40 protein inhibits, in a dose-dependent manner, the binding of 2×10^{-6} M of P40 Alexa488 (at more than 60% when it is used at 2×10^{-6} M) (cf. fig. 3).

Example 6: Among the TT, BB and rP40 carrier proteins, only the rP40 protein binds to the DCs.

Method

The tetanus anatoxin (TT) and BB (originating from the streptococcus G protein having affinity for human albumin) carrier proteins, and also the rP40 protein and the glycophorin A control protein were labeled with Alexa488 as described above. The binding of these molecules to the DCs was evaluated by flow cytometry as previously described. Briefly, 200 000 DCs are washed with FACS buffer and incubated in 50 µl of buffer in the presence of 10^{-6} M of each of the Alexa488-labeled proteins for approximately 1 hour at 4°C. After incubation, the cells are washed 3 times with FACS buffer, and then resuspended in 200 µl of this same buffer and analyzed by flow cytometry.

Result

At the concentration of 10^{-6} M, only rP40 binds to the dendritic cells. No binding of TT, BB and glycophorin is detected (cf. fig. 4).

Example 7: rP40 is internalized by the DCs

Method

200 000 DCs are washed with PBS-1% BSA buffer and resuspended, in a cone-bottomed 96-well plate, in 50 µl of PBS-BSA buffer (saline phosphate-bovine serum albumin buffer). The rP40-Alexa488 protein or the glycophorin-Alexa488 protein is then added at 2 x 10^{-6} M. Internalization kinetics are produced by incubating the cells with the Alexa-labeled proteins at 37°C for 15 minutes to 2 hours. A negative control for internalization is carried out under the same conditions, changing the following parameters: addition of 0.01% sodium azide to the PBS-BSA buffer and incubation of these cells with the Alexa-labeled proteins, at 4°C.

After incubation, the cells are then washed 3 times with PBS-BSA buffer, resuspended in 100 µl of this same buffer and then cytospun onto microscope slides. The slides are then analyzed by confocal microscopy.

Result

The observation of the cells incubated at 37°C with rP40-Alexa shows intracytoplasmic labeling which is detectable from 30 minutes and still observed after incubation for 2 h: a representative result, obtained after incubation for 1 h at 37°C is shown in figure 5B. Labeling of the membrane, but not intracytoplasmic labeling, is observed when the cells are incubated at 4°C with rP40 (cf. fig. 5A), whereas no labeling is observed in the presence of glycophorin-Alexa (after incubation at 4°C as at 37°C). The example of Alexa, a chemical molecule, demonstrates that any chemical molecule coupled to P40 can thus be delivered to antigen-presenting cells, including dendritic cells.